Chapter 5

Synergistic Effect of a Mixture of Benzimidazole and Iminoctadine Triacetate for the Preharvest Control of Benzimidazole-Resistant *Penicillium digitatum*, a Causal Agent of Citrus Green Mold in Japan

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Additional information is available at the end of the chapter

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Abstract

Green mold, caused by *Penicillium digitatum*, is the leading cause of citrus decay in Japan. Due to a ban on the post-harvest fungicide application in Japan, the preharvest application of benzimidazoles has been used and demonstrated good efficacy since 1971. A benzimidazole resistant *P. digitatum* strain was first isolated from a packinghouse in 1974, and more cases were reported in subsequent years. On the other hand, very few cases were reported from the grove for two decades. However, by the mid-1990s, when the field incidences of benzimidazole resistant strain started to increase, the effect of benzuimidazoles became unstaible. An alternative to a benzimidazoles, iminoctadine triacetate, exhibited good antifungal activity against *P. digitatum in vitro*, but its efficacy was inconsistent in the field. We examined the efficacy of a mixed application of iminoctadine triacetate and benzimidazoles against each fungicide by itself based on five years of data from multiple locations. The results indicated a synergistic suppression on green mold, where the efficacy of the mixture was consistently greater than treatments with either fungicide alone. The improved efficacy was considered acceptable for a practical use by the industry, and lead to a development of a pre-mixed commercial product, Beftopsin flowable in 2006.

Keywords: benzimidazole, benzimidazole-resistant, citrus green mold, iminoctadine triacetate, preharvest control, synergistic effect

1. Introduction

Postharvest diseases of citrus, particularly on the Satsuma mandarin orange (*Citrus unshiu*), including two very popular cultivars the mid-to-late-maturing citrus (Siranuhi; ((*C. unshiu* × *C. cinensis*) × *C. reticulata*) and Kiyomi-tangora (*C. unshiu* × *C. cinensis*)), cause serious problems at the production, distribution, and retail in Japan. Nowadays, Japanese consumers have very high expectations when it comes to food safety, and they have almost zero tolerance toward "rot" and "mold" on Satsuma mandarine oranges or any other fruit crops. Moreover, the identity of the production location (it could be as big as a region or as small as a town or individual farm) is often used as a part of marketing tools to ensure the quality of agricultural commodities in Japan. For example, it is not uncommon to see a picture of the grower on the package of fruits or vegetable. This works in favor, if the production location is producing high-quality products, but on the other hand, even a single rotten fruit in a shipment box can result in an unfavorable evaluation directly toward the production location. This may result not only in the price reduction of all fruits shipped from this location but also in the worst-case scenario result in a halt of the entire transactions and shipments of fruits from the region associated with the location. Therefore, it is not an exaggeration to say that a single rotten fruit can affect the future of a whole production region. Thus, if any rotten fruits are included among those shipped to the market, the particular shipment is often not distributed. In order to achieve this level of stringent quality control, robust countermeasures against postharvest diseases are needed.

In this chapter, the problem of fungicide-resistant strains associated with the occurrence of citrus green mold (caused by *P. digitatum*) in Satsuma mandarin orange, which causes the worst damage among Japanese postharvest diseases, is discussed based on the data from a previous study conducted by the author [1]. Then, the effectiveness of a tank-mix combination of fungicides as a preventative application against citrus green mold is explained based on another study conducted by the author [2]. Some of data from these previous studies were reanalyzed to meet the current standard.

2. Postharvest diseases in citrus cultivated in Japan

Several postharvest diseases affect citrus fruits (**Figure 1**). Among these diseases, green mold (caused by *P. digitatum*) [3], sour rot (caused by *Geotrichum candidum*) [4], blue mold (caused by *Penicillium italicum*) [3], Aspergillus rot (caused by *Aspergillus niger*, etc.) [5, 6], and anthracnose (caused by *Colletotrichum gloeosporioides*) [7] cause the greatest damage. Damage by Aspergillus rot of Satsuma mandarin orange becomes the serious problem in vinyl greenhouse in which the temperature may rise to more than 30°C in high summer season between July and September. Outbreak of sour rot is common in very early maturing Satsuma mandarin orange (VEMS) that is harvested in September when temperature is still high. Anthracnose is a common issue with very early maturing and early maturing Satsuma mandarin orange, which are harvested during the late-September to mid-October. The mid-maturing Satsuma mandarin orange or mid-to-late-maturing citrus are usually harvested during the late-November

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to mid-January and then stored in a room temperature (5–15°C) until shipment. When these fruits are stored for more than 3 months, Phomopsis stem-end rot (caused by *Diaporthe citri*) [8], whisker mold (caused by *Penicillium ulaiense*) [9], and Rhizopus rot (caused by *Rhizopus stolonifer*) [10] may occur. When it rained heavily or the tropical storm (typhoon) hit the production area before the harvest period, brown rot (caused by *Phytophthora* spp.) [11, 12] may occur frequently. Green mold and blue mold become the problem in all the Satsuma mandarin oranges and the mid-to-late-maturing citruses. Green mold tends to appear as fruits matured, and during storage period, and rate of blue mold outbreak can be very high during the middle to the late period of the storage, e.g., after 3–4 months in the storage.

Figure 1. Symptoms of citrus fruit rots mainly occurred in Japan; A: green mold caused by *Penicillium digitatum*, B: green mold (left) and blue mold (right) caused by *Penicillium italicum*, C: blue mold, D: whisker mold caused by *Penicillium ulaiense*, E: aspergillus rot caused by *Aspergillus niger*, F: sour rot caused by *Geotrichum candidum*, G: anthracnose caused by *Colletotrichum gloeosporioides*, H: brown rot caused by *Phytophthora palmivora*, I: black rot caused by *Alternaria citri*, and J: rhizopus rot caused by *Rhizopus stolonifer*.

Excluding anthracnose and brown rot, each of these pathogens infects plants through wounds on rind tissues, causing the fruit to rot. Development of anthracnose can be promoted by a damage on rind tissues, but wounds were not necessary to cause infection. The increase in problems associated with postharvest diseases of citrus in recent years is partially due to a strong consumer preference for high sugar and low acid contents. This demand has driven the production of ripe fruits with a thinner, bruise-prone rind. Because many postharvest citrus diseases take advantage of damages on rind tissues, the prevention of postharvest diseases on ripe fruits becomes more difficult.

3. Factors influencing the occurrence of postharvest diseases and prevention

The occurrence of postharvest diseases, such as green mold, is related to three major factors: (i) ripe fruit that rots easily, (ii) the presence of pathogens and its infection condition, and (iii) insufficient efficacy of fungicides. Several environmental and cultivation conditions affect these factors (**Figure 2**). The market demand for riper fruit results in more green mold susceptible fruits; furthermore, these fruits are more susceptible to damage to the surface of fruits, which can be caused by rough handling, contaminations in a harvest container such as dead twigs, cut peduncles, and very small pebbles [13] or by peduncles attached to fruits that were cut too long [14].

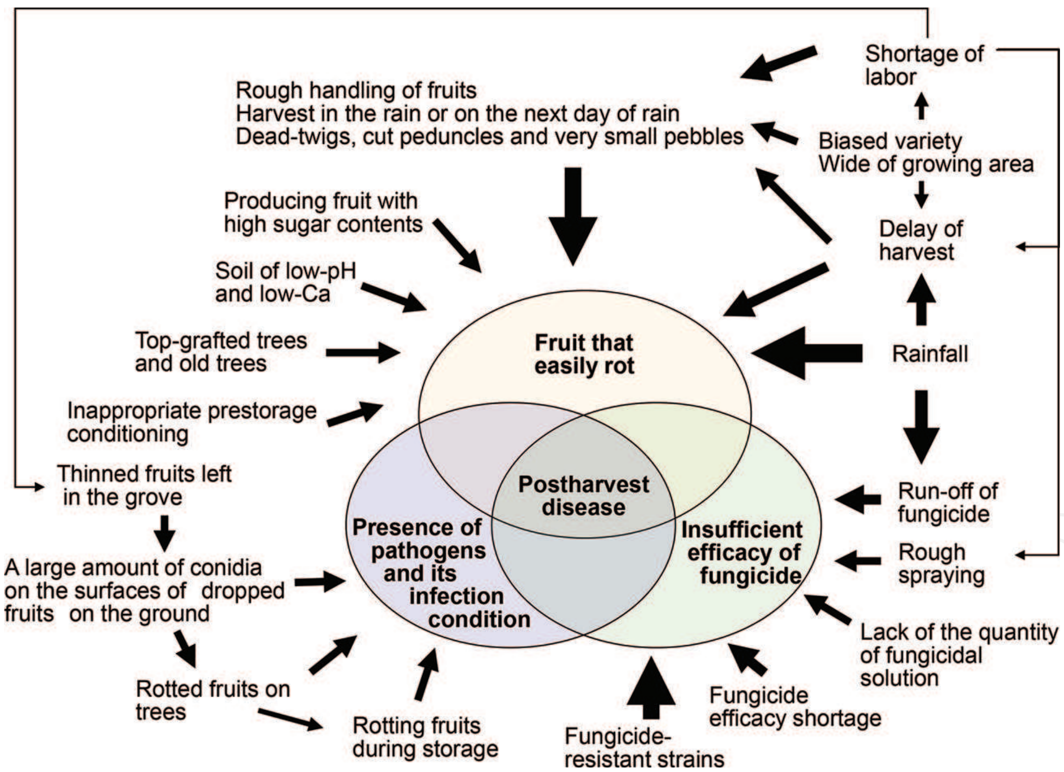

Figure 2. Factors influencing the occurrence of postharvest disease, especially citrus green mold in Japan.

It is safe to assume *P. digitatum* is a common pathogen in groves; thus, we may not have means of control over the presence of the pathogen; however, conditions to promote production of conidia that differ among groves may also differ among locations. For example, if sanitation measurements were not practiced, thinned fruits and fruits dropped by the animal and insect

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feedings can be the inoculum source, which can produce a large number of conidia in early autumn prior to harvest. Insufficient efficacy of fungicides maybe due to the emergence and increase of fungicide-resistant strains, lack of fungicide application and/or coverage, compromised spray coverage due to precipitations, and/or uneven adhesion of fungicides.

Presence of all three of these major factors is required for fruit rot development. For example, even if the surface of a fruit is damaged by rough handling, the fruit would not rot in the absence of a pathogen. Even if a damaged fruit is exposed to a pathogen, an effective fungicide would prevent rot. Therefore, if the effect of one of these three factors can be eliminated, postharvest diseases can be prevented. However, it is difficult to completely eliminate any of these factors because not only these three factors are influencing each other, but also each is highly depended on so many other external factors to form complex relationships (**Figure 2**). Therefore, rather than aiming for the elimination, it is important to aim for sufficient reduction of these three factors so that incidences of rotten fruits can be suppressed.

4. Basis for the implementation of cultural measures against postharvest diseases

4.1. Relationship between precipitation and postharvest diseases

Precipitation during harvest sharply increases the number of rotten fruits both at harvest and postharvest [15]. Precipitations not only make fruits more susceptible to rot [16], but it also washes fungicides off plants [17] to limit their function. In addition, many plant pathogens require water for their dissemination and penetration into plant tissues. However, when plants are grown outdoors, precipitation cannot be artificially controlled; thus, other than site selection and cultural practices to increase airflow in groves, our options against the precipitation are very limited.

4.2. Relationship between fruit cultivar, tree age, and postharvest diseases

Based on an analysis of fruits shipped from approximately 3200 groves, postharvest diseases tend to occur more frequently with fruits harvested from older trees than relatively young trees, irrespective of cultivar. The rate of rotten fruits did not differ between seedlings and top-grafted trees younger than 30 years but was higher with top-grafted trees 30 years or older [15]. These findings indicate the influence of age and type of grafting, and stricter countermeasures are probably necessary for top-grafted grove of 30 years or older. The other potential tools for green mold management may be derived from understandings of tree properties and soil conditions that promote the occurrence of rotten fruits, but further researches are needed.

4.3. Importance of the source of infection and the control of disease via the removal of the source

The quantity of spores from green mold fungus scattered in groves can be substantially reduced by removing fruits affected by green mold prior to the harvest. By thinning fruits and removing all fruits with sign of spore formation prior to the harvest, the occurrence of rotten fruits can be reduced [13]. For example, following the final thinning of fruits, some growers completely remove debris from their groves. This is a cultural measure that needs to be actively promoted in the future.

5. Importance of fungicide control

Damage to the surface of fruits, which often happen during the processes of harvest, transportation, and sorting, is the only means of infection for *P. digitatum* and *P. italicum*. Careful handling of fruits minimizes damage and can prevent rot. However, despite the awareness of this issue and availability of instructions for the careful handling of fruits to pickers, good cultural practices are not commonly implemented. All the tree fruit harvests in Japan are performed by manual labor, and the lack of skilled labor when they are needed is generally the cause for rough handling. It can be due to a lack of manpower to cover large growing areas and/or uneven distribution of workload owing to a concentrated production of a specific market-favored variety. Furthermore, autumn is a season for tropical storms in Japan. These adverse weather conditions during or close to the harvest period can cause delays, and then force harvesting to be completed within an extremely short time frame, resulting in very little care during fruit handling. Therefore, to account for realistic conditions at harvest time, control measures must be developed based on the assumption that the rind will be damaged; hence, preharvest fungicide sprays become important.

6. Fungicide use to prevent postharvest diseases and the development of resistant strains

The fungicide with a superior effect against the green mold and the blue mold has not existed until the latter half of 1960s. Thus, by 1971, soon after the introduction of benzimidazoles, it became common to apply a benzimidazole (benomyl or thiophanate-methyl) immediately prior to harvest in Japan. In Japan, the use of fungicide to harvested fruits is prohibited by the Agricultural Chemicals Control Act. Therefore, unlike countries in which harvested fruits are drenched or dipped in fungicides in packinghouses in order to prevent the rotting of fruits during storage or transportation [3,18–20], the only chemical control measure available in Japan is fungicides that are sprayed in groves prior to harvest. The preharvest spray of benzimidazoles was shown to have a favorable suppressive effect on many postharvest diseases, particularly green mold [21–30]. Furthermore, iminoctadine triacetate [31, 32], which has a different mode of action from that of benzimidazoles, became available in 1985. Presently, seven preharvest fungicides have been registered to prevent postharvest diseases in Japan, but these two fungicides (i.e., iminoctadine triacetate and benzimidazoles) are the only ones in use. Compared to other mode of action groups, their effects are consistent, and costs are relatively low. However, compared to many other diseases of citrus or other crops that are typically managed using four or five modes of action, the use of just two fungicides for citrus postharvest disease increases the risk of resistant strain development.

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Benomyl, (methyl 1-(butylcarbamoyl) benzimidazol-2-yl-carbamate, is a systemic benzimidazole fungicide discovered by DuPont in the early 1960s (rates and other information are summarized in **Table 1**). It prevents cell division in sensitive fungi by binding to tubulin and inhibiting the formation of spindle fibers at metaphase and is highly active at low concentrations against spore germination and mycelial growth. Further, locally systemic properties enable the fungicides to penetrate the host tissue and provide post-infection curative action. It was first registered under the brand name Benlate (50% wettable powder) by DuPont in Japan in 1971 and Sumitomo Chemical Co., Ltd. (Tokyo, Japan) acquired the business in 2002. Benomyl is a very broad-spectrum fungicide with low phytotoxicity and controls many fungi in the classes Ascomycetes, Deuteromycetes, and Basidiomycetes. It has long been used for wide range of crop groups, notably for citrus as a postharvest diseases control agent with its strong rainfastness and residual activity.

Generic name	Trade name	FRAC code	Active ingredient (%)	Company, city, country	Rate applied (μg/mL)
Benomyl	Benlate wettable powder	1	50.0	Sumitomo Chemical Co., Ltd., Tokyo, Japan	125
Thiophanate-methyl	Topsin-M wettable powder	1	70.0	Nippon Soda Co., Ltd., Tokyo, Japan	350
Iminoctadine triacetate	Befran 25 liquid	M7	25.0	Nippon Soda Co., Ltd., Tokyo, Japan	125

The spray volume for preharvest application was 500 L/10 a.

Table 1. Common name of each fungicide tested, their corresponding trade names, active ingredient percentages, manufacturers, and preharvest application rates.

Thiophanate-methyl, dimethyl 4,4′-(*o*-phenylene) bis(3-thioallophanate), is a methyl benzimidazole carbamate fungicide with preventive and curative effects (FRAC group 1). Thiophanate-methyl is converted to MBC (methyl benzimidazol-2-ylcarbamate) on the plant surface and in tissues, and MBC inhibits β-tubulin assembly during mitosis in fungi. Its spectrum includes Basidiomycota, Ascomycota, and imperfect fungi. It was first registered in Japan in 1971 under the brand name Topsin-M (70% wettable powder; Nippon Soda Co. Ltd. (Tokyo, Japan) as a pre-harvest spray targeting blue mold, green mold, and stem-end rot on citrus. Long-lasting effects are high because they exhibit penetrative and systemic activities.

Iminoctadine triacetate, 1,1′-iminodi (octamethylene) diguanidinium triacetate, is a bis-guanidine with preventive effects (rates and other information are summarized in **Table 1**). It is classified as a multi-site contact activity fungicide and is generally considered to have a low resistance risk according to the FRAC (group M7). Its mode of action involves cell membrane transport and sterol biosynthesis at different sites via C14-demethylase in sterol biosynthesis inhibitors. Owing to these unique mechanisms of action, it is a good tool to manage resistance to fungicides with various modes of action, e.g., benzimidazoles, dicarboximides, C14-demethylase inhibitors, Qo inhibitors, and succinate dehydrogenase inhibitors. Its spectrum includes Ascomycota and imperfect fungi, and it inhibits spore germination, germ tube elongation, and appressorium and infectious hypha formation in the life cycle of pathogens. It was first registered in Japan in 1983 under the brand name Befran (25% liquid; Nippon Soda Co., Ltd.) as a preharvest spray targeting blue mold, green mold, Alternaria rot, and sour rot on citrus. However, the efficacy of this chemical on stem-end rot is lower than benzimidazoles, which shows a superior effect. Since it is a contact fungicide, spraying just before harvest is optimal.

6.1. Occurrence and spread of green mold fungus resistant to benzimidazoles

The extensive usages of benzimidazoles have led to the development of benzimidazole-resistant strains (BRS) of *P. digitatum* and *P. italicum*, which was first discovered in Kanagawa and Shizuoka prefectures in 1974 [33, 34]. Since then, BRS have been found in Saga prefecture [35] and other major producing areas [36, 37]. Concern about a decline in control effects owing to BRS has prompted many studies.

6.2. Despite BRS incidences, the reduction of benzimidazoles' efficacy was not reported prior to 1990s

These studies found that the incidence of BRS in groves sampled immediately after spraying with was either very low or nondetectable [37, 38]. Among early maturing Satsuma mandarin orange, which is shipped immediately after harvest or after a short period of storage, there was no decrease in control efficacy even after the development of BRS [37, 38]. In some varieties, such as mid-maturing Satsuma mandarin orange and mid-to-late-maturing citrus that are shipped after storage, the incidence of BRS tends to gradually increase during storage [35]. However, if the damages on the fruit surface are limited, the disease incidence does not increase quickly [37]. Moreover, there were reports of BRS in producing areas nationwide [39], but these occurrences were not severe enough to cause actual damage. Thus, despite reported cases of BRS, the efficacy of benzimidazoles was considered to be maintained at the acceptable level [39]. Therefore, it was concluded that the efficacy of benzimidazole-based chemical management was not decreased by BRS [39].

6.3. BRS is already present in groves prior to harvest

High numbers of BRS isolates were observed after an outbreak of green mold in harvested greenhouse Satsuma mandarin orange fruits in Saga prefecture in late August of 1993. Subsequently, a survey of fruits from packinghouses in Saga was conducted [1] and resulted in a large number of thiophanate-methyl–resistant strains (**Table 2**). Without the host, *P. digitatum* conidia can survive in the packinghouse for 1 month or more in the winter; however, they can only survive for about 5 days during summer months [40]. Thus, it is less likely that packinghouses were contaminated when new crops were brought in a packinghouse in late August to mid-October. This further confirms that resistant fungi were already present at the harvest whether they came from greenhouses or groves.

Location of packinghouse	Source of isolate[2]	Rate of resistant strains[3]	Number of strains for each MIC range (μg/mL)		
			≤0.78	1.56–200	>1600
Hamatama	SGPG	14/14	0	0	14
Chinzei	VEMS	5/5	0	0	5
Tara	VEMS	11/11	0	0	11
Omachi	VEMS	13/14	1	0	13
Ogi	VEMS	5/15	10	2	3
Total		48/59 (100)[4]	11 (19)	2 (3)	46 (78)

[1] The investigations were performed from end August to beginning of October in 1993. Original data for the table are from [1], adapted with a permission from the authors.

[2] SGPG, Satsuma mandarin orange grown in a vinyl greenhouse; VEMS, very early maturing Satsuma mandarin orange.

[3] No. of resistant strains (MIC > 1.56 μg/mL)/(no. of strains tested).

[4] Values in parentheses are the percentages of the total strains in the category.

Table 2. Thiophanate-methyl sensitivity of *Penicillium digitatum* strains isolated from Satsuma mandarin orange at various packinghouses in Saga prefecture in Japan.[1]

6.4. Confirming cases of decreased control effects by resistant strains

In order to determine the presence of BRS, a series of experiments were conducted from 1993 to 1995 [1]. Fruits were artificially damaged just before harvest to promote development of green mold. Throughout the experiment, benzimidazoles were not sprayed. *P. digitatum* was then isolated from diseased fruits and examined for sensitivity to a benzimidazole (Thiophanate-methyl). Overall, highly resistant strains were detected at a high frequency at vinyl greenhouse (**Table 3**) and at grove (**Table 4**).

Year investigated	Location of grove sampled	Rate of resistant strains[1]	Number of strains for each MIC range (μg/mL)		
			≤0.78	1.56–100	>1600
1993	Hamatama-1	15/18	3	0	15
	Hamatama-2	23/23	0	0	23
	Total	38/41 (93)	3 (7)	0 (0)	38 (93)
1994	Hamatama-1	15/28	13	0	15
	Hamatama-2	15/26	11	0	15
	Total	30/54 (56)	24 (44)	0 (0)	30 (56)
1995	Hamatama-1	18/52	34	0	18
	Hamatama-2	11/32	21	0	11
	Total	29/84 (35)	55 (66)	0 (0)	29 (34)

[1] Number of resistant strains (see **Table 2**)/total number of strains tested. Values in parentheses are the percentages of the total for each category. Original data for the table are from [1], adapted with a permission from the authors.

Table 3. The number of thiophanate-methyl resistant *Penicillium digitatum* strains isolated from Satsuma mandarin orange grown in plastic greenhouses.

Year investigated	Location of grove sampled	Rate of resistant strains[1]	Number of strains for each MIC range (μg/mL)		
			≤0.78	1.56–200	>1600
1993	Tara	22/25	3	10	12
	Oura	40/46	6	7	33
	Kashima	26/26	0	5	21
	Ogi	10/10	0	6	4
	Yamato	24/38	14	11	13
	Kagami	29/39	10	9	20
	Total	151/184 (82.1)	33 (17.9)	48 (26.1)	103 (56.0)
1994	Tara	2/9	7	0	2
	Oura	2/24	22	0	2
	Kashima	6/13	7	3	3
	Ogi-A	7/14	7	0	7
	Hamatama-A	14/23	9	3	11
	Total	31/83 (37.3)	52 (62.7)	6 (7.2)	25 (30.1)
1995	Tara	5/15	10	0	5
	Oura	11/16	5	7	4
	Kashima	5/12	7	1	4
	Yamato	0/12	12	0	0
	Kitahata	18/45	27	0	18
	Ogi-B	8/29	21	0	8
	Hamatama-A	2/22	20	0	2
	Hamatama-B	3/13	10	0	3
	Kyuragi	2/17	15	0	2
	Total	54/181 (29.8)	127 (70.2)	8 (4.4)	46 (25.4)

[1] Number of resistant strains (see **Table 2**)/total number of strains tested. Values in parentheses are the percentages of the totals for each category. Fruits were artificially wounded prior to applications of treatment. Original data for the table are from [1], adapted with a permission from the authors.

Table 4. The number of thiophanate-methyl resistant *Penicillium digitatum* isolated from Satuma mandarin orange grown in groves.

Furthermore, another experiment was conducted in a total of six groves to examine the effect of wounding and fungicide treatments [1]. Three groves, each consisted of the one cultivar, were examined in 1994 and 1995. The experimental design was a split-plot design where the main block consisted of two wound treatments (artificial wounding or not) and the subplots consisted of three fungicide treatments (thiophanate-methyl, iminoctadine triacetate, and non-treated control). The experimental unit was a fruit, and 50 fruits per subplot were examined. Based on the data from this study [1], a generalized linear mixed model (GLMM) [41] (PROC GLIMMIX, SAS ver 9.4, SAS Institute, Cary, NC, USA) was utilized to reexamine the effects of grove, wounding, and fungicide treatments on the probability of green mold disease incidence per fruit. Grove, wounding, and fungicide were considered as a fixed effect, and a logit was used as a link function.

The number of rotted fruit ranged from 0 to 40 out of 50 fruits examined per subplot (**Table 5**). The results from a GLMM showed significant grove ($F = 37.5$, $P < 0.01$), wounding ($F = 117.8$, $P < 0.01$), fungicide ($F = 12.1$, $P < 0.01$), and wounding × fungicide interaction ($F = 5.6$, $P = 0.01$). The interaction between wounding and fungicide treatment showed that all the fruits with wounding treatment resulted in higher probabilities ($P \leq 0.05$) of rotted fruit than nonwounded fruits; however, the effect of fungicide treatment differed between wounding and nonwounding treatments. With wounding treatment, fruits treated with thiophanate-methyl and nontreated control resulted in significantly higher probabilities of rotted fruit ($P < 0.05$) than iminoctadine triacetate treatment (**Table 6**). On the other hand, with nonwounding treatment, both thiophanate-methyl and iminoctadine triacetate treatments resulted in significantly lower probabilities of rotted fruit ($P < 0.05$) than nontreated control (**Table 6**). The results indicated the importance of a wounding event to the development of green mold, as well as the lack of efficacy provided by thiophanate-methyl.

Treatment	**Fungicide and rate**	**Number of rotted fruit/number of fruit investigated**					
		Kashima	**Ogi-1**	**Oura**	**Kyuragi**	**Tara**	**Ogi-2**
No-wound	Control	0/50	14/50	0/50	4/50	2/50	0/50
	Thiophanate-methyl 350 μg/mL	0/50	3/50	1/50	1/50	0/50	0/50
	Iminoctadine triacetate 125 μg/mL	0/50	1/50	0/50	0/50	1/50	0/50
Wound[1]	Control	12/50	40/50	7/50	34/50	8/50	4/50
	Thiophanate-methyl 350 μg/mL	15/50	15/50	6/50	40/50	38/50	5/50
	Iminoctadine triacetate 125 μg/mL	5/50	4/50	3/50	30/50	7/50	0/50
	Number of resistant strains	**(6/13)**[2]	**(7/14)**	**(2/24)**	**(2/17)**	**(5/15)**	**(8/29)**

[1] The fruits were damaged at three places per 1 fruit to 2 mm depth of the rind of a fruit using the inoculation tool which three setting pins were bundled up. Original data for the table are from [1], adapted with a permission from the authors.
[2] Boldfaced ratios: number of resistant strains (MIC ≥ 1.56 μg/mL, see **Table 2**)/number of strains tested. Kashima, Ogi-1, and Oura were tested in 1994, and Kyuragi, Tara, and Ogi-2 were tested in 1995.

Table 5. The efficacy of a preharvest application of thiophanate-methyl and iminoctadine triacetate on green mold fruit rot in groves of very early maturing Satsuma mandarin orange, and the detection frequency of BRS from each grove.

These findings differed from previous results indicating that the incidence of BRS is extremely low in groves prior to the harvest [37, 38]. When harvesting greenhouse Satsuma mandarin orange, very early maturing Satsuma mandarin orange, and early maturing Satsuma mandarin orange, BRS were present at a high rate and thus many cases of postharvest disease were observed.

Wound	Fungicide and rate	Mean probability of rotted fruit[1]	Standard error
No-wound	Control	0.042 C	0.010
	Thiophanate-methyl 350 μg/mL	0.010 D	0.004
	Iminoctadine triacetate 125 μg/mL	0.004 D	0.003
Wound	Control	0.320 A	0.031
	Thiophanate-methyl 350 μg/mL	0.380 A	0.033
	Iminoctadine triacetate 125 μg/mL	0.118 B	0.019

[1] The analysis of wound and fungicide treatment effects on the mean probability of rotted fruit was conducted using a generalized linear mixed model (PROC GLIMMIX, SAS, ver. 9.4). A significant wounding and fungicide treatment interaction was observed ($F = 5.6$, $P = 0.01$). The estimated mean probabilities of rotted fruit and standard errors from the model are shown in the table. The different letters following two numbers indicate these two treatments are significantly ($P \leq 0.05$) different from each other, based on Fisher's LSD. The original data from [1] were re-analyzed with a permission from the authors.

Table 6. The effect of wounding and preventative application of thiophanate-methyl and iminoctadine triacetate on the probability of green mold fruit rot in groves of very early maturing Satsuma mandarin orange using the pooled data from **Table 5**. Values followed by different letters differed significantly.

The high rate of BRS during the harvest of greenhouse Satsuma mandarin orange and very early maturing Satsuma mandarin orange may be explained by the continuous use of fungicides over 20 years. BRS and benzimidazole-sensitive strains of *P. digitatum* did not differ with respect to virulence, lesion expansion, and sporulation [38]. This lack of competitive fitness cost most likely promoted increase in the number of BRS under continuous uses of benzimidazoles. In addition, recent increases in the use of the vinyl-greenhouse for production of Satsuma mandarin oranges changed seasonal availability of *P. digitatum* spores. With the traditional outdoor growing practices, Satsuma mandarin orange fruits were not ripe enough for *P. digitatum* to produce spores during the hot summer. However, the vinyl-greenhouse system allows a long harvest period of Satsuma mandarin oranges from April to September; thus, when very early maturing Satsuma mandarin orange in the outdoor field are ready for harvest in the mid-September, they will be exposed abundant spores produced in the vinyl-greenhouse production. Moreover, these isolates from the vinyl-greenhouse production are most likely exposed to a benzimidazole; thus, fruits from the outside field can be exposed to a high number of BRS even prior to the application of a benzimidazole.

6.5. Increasing of BRS after benzimidazole spraying in groves

Another experiment was conducted in 1995 using three groves where detection frequencies of BRS were examined before and after application of fungicide treatments [1]. Prior to the experiment, the detection frequencies of resistant strains were low (12–33%, **Table 7**) and not significantly different among groves ($\chi^2 = 2.3$, $P = 0.3$, reanalyzed using the data from [1], PROC FREQ, SAS ver. 9.4) (**Table 7**). Five trees were treated with each treatment, and fruits with symptoms of green mold were examined for the detection of BRS. The effect of grove and fungicide treatment on the probability of BRS detection after fungicide treatment application was reexamined using a GLMM with a logit as the link function. A significant effect of fungicide on the probability of detecting BRS was found (F = 20.9, P = 0.01; **Table 8**), but the grove effect was not significant (F = 1.9, P = 0.26). Thiophanate-methyl treatment resulted in a higher probability of detecting BRS than that of iminoctadine triacetate or nontreated control treatments. The high recovery rate of BRS despite the small number of samples indicates the abundance of BRS conidia in these groves.

Grove[2]	Fungicide	Rate applied	Detection ratio of resistant strains[3]	
			Before spraying[4]	After spraying
Kyuragi	Thiophanate-methyl 70% wettable powder	350 μg/mL	–	10/12
	Iminoctadine triacetate 25% soluble concentrate	125 μg/mL	–	1/16
	No fungicide	–	2/17 A	0/10
Tara	Thiophanate-methyl 70% wettable powder	350 μg/mL	–	31/31
	Iminoctadine triacetate 25% soluble concentrate	125 μg/mL	–	2/11
	No fungicide	–	5/15 A	4/10
Ogi-2	Thiophanate-methyl 70% wettable powder	350 μg/mL	–	8/8
	Iminoctadine triacetate 25% soluble concentrate	125 μg/mL	–	0/1
	No fungicide	–	8/29 A	1/4

[1] Investigation was performed in 1995. Original data for the table are from [1], adapted with a permission from the authors.

[2] See **Table 5** regarding the preventive effect of fungicides in the groves.

[3] The number of the resistant strains (see **Table 2**)/the number of strains tested. Fruits of very early maturing Satsuma mandarin orange were artificially wounded to promote fungal infection.

[4] There is no significant effect of grove on the detection ratio of resistant strain (χ^2 = 2.3, P = 0.3, PROC FREQ, SAS ver. 9.4) prior to the application of fungicide treatment. Values followed by same letters show not significantly different.

Table 7. Detection frequency of thiophanate-methyl–resistant strains of *Penicillium digitatum* isolated at before and after the fungicide treatment from fruits of very early maturing Satsuma mandarin orange.[1]

Fungicide and rate	Mean probability of BRS[1]	Standard error
Control	0.327 B	0.102
Thiophanate-methyl 350 μg/mL	0.961 A	0.031
Iminoctadine triacetate 125 μg/mL	0.105 B	0.067

The analysis of fungicide treatment effect on the mean probability of BRS was conducted using a generalized linear mixed model (PROC GLIMMIX, SAS, ver. 9.4). The expected mean probabilities of BRS and standard errors from the model are shown. The different letters following two numbers indicate these two treatments are significantly ($P \leq 0.05$) different from each other, based on Fisher's LSD. The original data from [1] were re-analyzed with a permission from the authors.

Table 8. The effect of application of thiophanate-methyl and iminoctadine triacetate on the probability of detection ratio of BRS, using the pooled data from **Table 7**.

Results from these series of experiment consistently show the lack of efficacy provided by thiophanate-methyl (**Tables 5** and **6**), as well as the high probability of detecting BRS from thiophanate-methyl–treated fruits (**Tables 7** and **8**). These results triggered growers to use iminoctadine triacetate, which also shown to be effective against green mold in our study (**Tables 5** and **6**).

6.6. Problems with iminoctadine triacetate

Although the use of iminoctadine triacetate shows strong antifungal activity *in vitro* against *P. digitatum* and *G. candidum*, its efficacy on green and blue mold in the field is shown to be inconsistent. As shown in our study, its efficacy in the field may not be sufficient to achieve a high level of protection, especially when fruit surfaces are damaged (**Table 6** and **Figure 3**). The same concern was discussed in studies by Koga et al. [42] and Miyoshi et al. [43].

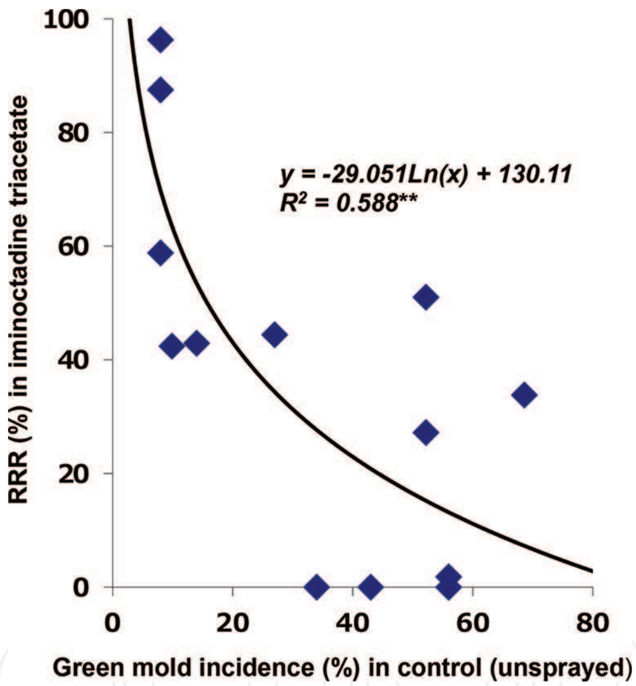

Figure 3. Relationship between the occurrence of green mold in control conditions and the preventive effect of iminoctadine triacetate. Preventive effects are based on the relative risk reduction (RRR).

7. Tank-mix combination of fungicides for resistant strains

As discussed above, there are loss of efficacy provided by benzimidazoles, and the efficacy of iminoctadine triacetate, are found to be relatively inconsistent. Alternative fungicides to replace these materials are in need; however, there is no such alternative available in Japan at 1990s.

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Combining fungicides with different modes of action can be more effective compared to the separate use of a single fungicide [44–51]. There are four main reasons for considering the use of fungicide mixtures [52, 53]. (1) Broadening the target spectrum: If the aim is to control two target pathogens that differ in sensitivity to fungicide modes of action with one application, it can be useful to spray mixtures of fungicides. (2) Improved disease control: if the target pathogen is susceptible to two modes of action, a use of two effective modes of action should increase the efficacy. (3) As an insurance against resistance: if a certain population of the target pathogen develops resistance to one of the mixture components, the other mode of action in the mix can act against the resistant population. (4) Resistance management: with a combination of reasons #2 and #3, the rate of resistance development among pathogen populations can be delayed, thus, the effective life of a fungicide can be extended. For our situation, the mix of a benzimidazole and iminoctadine triacetate is considered because both are still somewhat effective against green mold. For example, benzimidazoles sometimes show preventative effects even when resistant strains are detected at a high frequency, as shown in one of our experiments (**Table 5**; Ogi-1). In addition, antifungal activity of iminoctadine triacetate *in vitro* against *P. digitatum* is high [32], and our experiment also showed a significant reduction of green mold (**Table 6**). Thus, a series of experiments were conducted to examine the efficacy of a mixture of a benzimidasole and iminoctadine triacetate [2].

7.1. Improved effects using a tank-mix combination of benzimidazole and iminoctadine triacetate

Table 9 shows the results of the effects of the combined use of 125-ppm benomyl and 125-ppm iminoctadine triacetate on incidence of green mold from different studies [2]. The preventive effect of treatments was compared based on relative risk reduction (RRR). RRR is the percent reduction in risk in the treated group (e.g., benomyl application) compared to the control group (e.g., no application). RRR = (1 − risk ratio: RR) × 100. RRR values showed that a tank-mix combination of benzimidazole and iminoctadine triacetate improved the preventive effect substantially in 11 of 12 studies. Using a meta-analysis, the total relative risk ratio was 0.23 (95% confidence interval, 0.15–0.33), as shown in **Figure 4**. Including the error range, the risk ratio was 1.0 or less; thus, the onset of disease is significantly less than that in unsprayed plants (by approximately 15–33%).

Incidences of diseased fruits in unsprayed grove block was not associated with those in the block of the combined spray, as shown in **Figure 5** (R^2 = 0.171). In the other words, even with the cases with higher incidences of diseased fruits in the unsprayed grove block, the preventive effect in the combined-sprayed area did not decrease, indicating that the efficacy of the combined spray was not affected by the background level of green mold rot. Moreover, regardless of the BRS detection frequency, a stable preventative effect was obtained (**Table 9**). Harmful effects, such as brown discoloration and/or delayed color development, using the combined benomyl and iminoctadine triacetate spray were not confirmed in any of the experiments.

Year	Study	Spray timing (days to harvest)	Rate of BRS[1]	Fungicide and rate: Benomyl 125 μg/mL		Iminoctadine triacetate 125 μg/mL		Iminoctadine triacetate 125 μg/mL + benomyl 125 μg/mL		Incidence of control (%)[3]
				Incidence (%)	RRR (%)[2]	Incidence (%)	RRR (%)	Incidence (%)	RRR (%)	
1999	Ogi-1	21	4/15	55.0	1.8	55.0	1.8	8.0	85.7	56.0
1999	Ogi-2	14	4/15	18.0	67.9	60.0	0	3.0	94.6	56.0
1999	Ogi-3	7	4/15	7.0	87.5	65.0	0	3.0	94.6	56.0
2000	Tara	14	9/16	54.0	0	72.0	0	8.0	76.5	34.0
2000	Taku	7	6/10	23.0	46.5	52.0	0	4.0	90.7	43.0
2003	Taku-1	21	4/11	5.0	37.5	3.3	58.8	0.7	91.3	8.0
2003	Taku-2	14	4/11	7.7	3.8	0.3	96.3	1.7	78.8	8.0
2003	Taku-3	7	4/11	0.7	91.3	1.0	87.5	3.3	58.8	8.0
2004	Tara-1	7	–[3]	–[3]		25.6	51	11.5	78.0	52.2
2004	Tara-2	21	3/11	24.6	52.9	38.0	27.2	13.8	73.6	52.2
2004	Chinzei-1	21	–[3]	14.3	0	24.9	0	10.3	0	9.9
2004	Chinzei-2	7	–[3]	–[3]		5.7	42.4	2.9	70.7	9.9
2005	Ogi-1	7	3/11	22.0	18.5	15.0	44.4	14.0	48.1	27.0
2005	Ogi-2	7	2/14	10.0	28.6	8.0	42.9	4.0	71.4	14.0

Benzimidazole-resistant strain detection frequency before benzimidazole application; the number of the resistant strains/investigated strains.

The preventive effects are based on relative risk reduction (RRR). RRR is the percent reduction in risk in the treated group (thiophanate-methyl application) compared to the control group (no application). RRR = (1 − risk ratio: RR) × 100. Risk ratio is shown in **Table 6**. In evaluating the effect, a high RRR indicates high effectiveness.

Examination and investigations were not enforced. Original data in the table are from [2], adapted with a permission from the authors.

Table 9. Efficacy of preharvest application of benomyl, iminoctadine triacetate, and tank mix of two materials against green mold on very early maturing Satsuma mandarin orange. Harvested fruits were subjected to an artificial wounding treatment where fruits were rolled on a sloped concrete surface for 5 m.

Year	Study	Tank-mix Events	Tank-mix Total	Control Events	Control Total	RR	95%-CI	W(fixed)	W(random)
1999	**Ogi-1**	8	100	56	100	0.14	[0.07; 0.28]	7.9%	7.9%
1999	**Ogi-2**	3	100	56	100	0.05	[0.02; 0.17]	7.9%	5.5%
1999	**Ogi-3**	3	100	56	100	0.05	[0.02; 0.17]	7.9%	5.5%
2000	**Tara**	8	100	34	100	0.24	[0.11; 0.48]	4.8%	7.7%
2000	**Taku**	4	100	43	100	0.09	[0.03; 0.25]	6.1%	6.2%
2003	**Taku-1**	2	300	24	300	0.08	[0.02; 0.35]	3.4%	4.3%
2003	**Taku-2**	5	300	24	300	0.21	[0.08; 0.54]	3.4%	6.4%
2003	**Taku-3**	10	300	24	300	0.42	[0.20; 0.86]	3.4%	7.7%
2004	**Tara-1**	42	365	155	297	0.22	[0.16; 0.30]	24.3%	9.9%
2004	**Tara-2**	32	232	155	297	0.26	[0.19; 0.37]	19.3%	9.7%
2004	**Chinzei-1**	22	214	19	192	1.04	[0.58; 1.86]	2.8%	8.5%
2004	**Chinzei-2**	6	205	19	192	0.30	[0.12; 0.72]	2.8%	6.7%
2005	**Ogi-1**	14	100	27	100	0.52	[0.29; 0.93]	3.8%	8.5%
2005	**Ogi-2**	4	100	14	100	0.29	[0.10; 0.84]	2.0%	5.7%
Fixed effect model			**2616**		**2578**	**0.23**	**[0.20; 0.27]**	**100%**	**--**
Random effects model						**0.23**	**[0.15; 0.33]**	**--**	**100%**

Heterogeneity: I-squared=77.2%, tau-squared=0.3631, p<0.0001

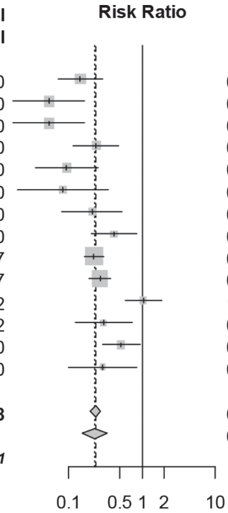

Figure 4. The forest plots represent the integrated meta-analysis [54–58] of the effect of the tank-mix application of benomyl and iminoctadine triacetate on green mold of very early ripening Satsuma mandarin orange in 14 field trials. Data were analyzed by a meta-analysis in a random effects model using the DerSimonian-Laird method [56, 59]. The meta-analysis was performed using the EZR [60] graphical user interface for R software (The R Foundation for Statistic Computing, version 2.14.0). Each gray square marks the value of the risk ratio (RR) [61] of each study, and the size of the square indicates the weight of each field trial. The horizontal line indicates the 95% confidence intervals of the effect estimate of individual studies. The diamond at the bottom of the graph shows the integrated risk ratio, and the width of the diamond shows the confidence intervals for the overall effect estimate.

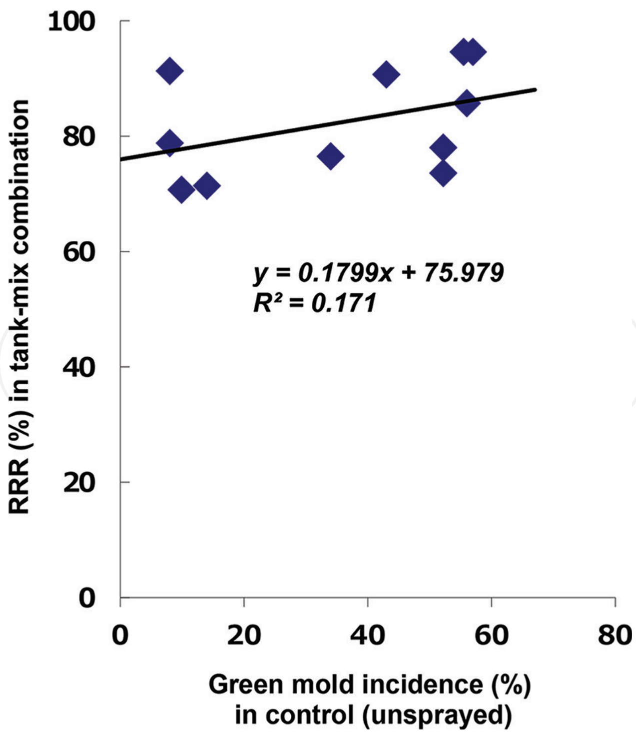

Figure 5. Relationship between the occurrence of green mold in the control and the preventive effect of the tank-mix combination of benomyl and iminoctadine triacetate.

In the comparison between the combined spray of benomyl and iminoctadine triacetate and the individual fungicides, the preventative effect increased greatly. Combined spraying with another benzimidazole, thiophanate-methyl, and iminoctadine triacetate, also substantially decreased the occurrence of rotten fruits (**Figure 6** and **Table 10**).

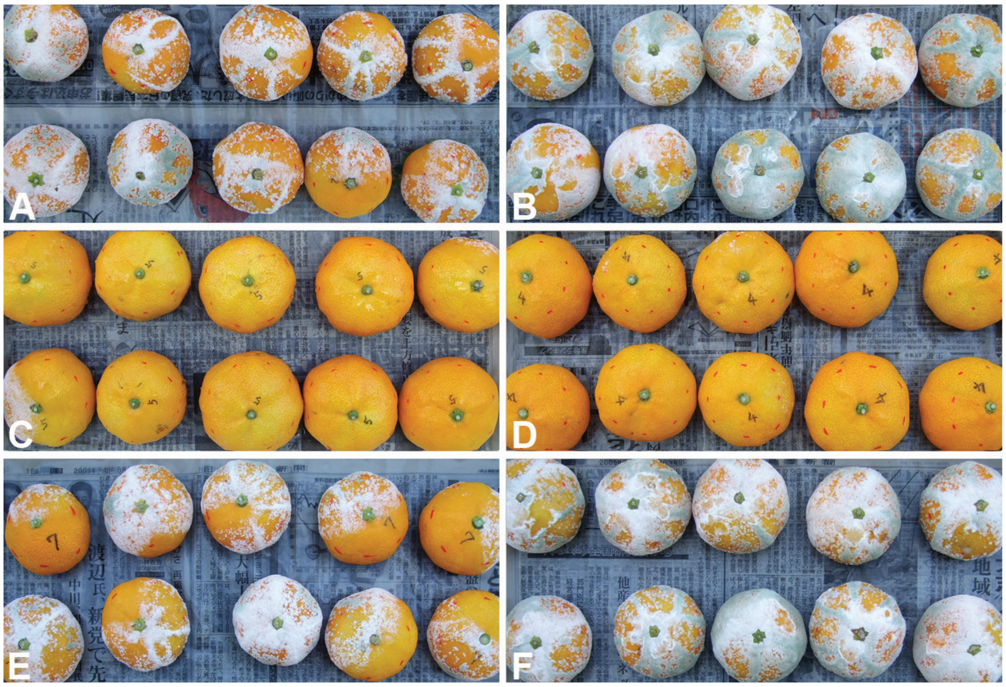

Figure 6. The efficacy of the tank-mix combination of benzimidazoles and iminoctadine triacetate for the control of green mold in very early ripening satsuma mandarin orange. A, benomyl 125 μg/mL; B, thiophanate-methyl 350 μg/mL; C, benomyl 125 μg/mL + iminoctadine triacetate 125 μg/mL; D, thiophanate-methyl 350 μg/mL + iminoctadine triacetate 125 μg/mL; E, iminoctadine triacetate 125 μg/mL; F, control. Fruits were harvested 3 days after spraying and artificially inoculated with a conidial suspension (10^6/mL) of benzimidazole-resistant *Penicillium digitatum* (EC50 of benomyl = 6.25 μg/mL, mutation in β-tubulin codon 200 of phenylalanine to tyrosine). Green mold incidence was evaluated after storage for 1 week under 98% relative humidity.

7.2. Synergistic effects of the tank-mix combination

The control effects of the combined spray were superior to the effects of each individual fungicide. Whether the effects were additive or synergistic was examined using the Colby method [62], which compared theoretical values based on an additive model and measured values. Effects are additive if the total efficacy of multiple fungicides is similar or equal to the sum of the efficacies of individual fungicide. Synergistic effects are inferred when a total efficacy of multiple fungicides is greater than the sum of the efficacies provided by two individual fungicides.

As shown in **Table 11**, eight out of 12 tests (excluding Taku-2 and Taku-3 (2003), Chinzei-1 (2004), and Ogi-1 (2005)) showed the (actual RRR/expected RRR) values larger than 1. Thus, the observed RRR value was greater than the theoretical RRR values, indicating the synergistic effect of the combined spray. In Taku-2 and Taku-3 (2003), good control was achieved in not only the combined treatment but also benomyl only and iminoctadine triacetate only

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Fungicide and rate	The number of the tests	Combined risk ratio (95% CI)[2]	
Benomyl 125 μg/mL	12	0.60	(0.41–0.89)
Iminoctadine triacetate 125 μg/mL	14	0.80	(0.62–1.04)
Benomyl 125 μg/mL + iminoctadine triacetate 125 μg/mL	14	0.23	(0.15–0.33)
Thiophanate-methyl 350 μg/mL + iminoctadine triacetate 125 μg/mL	11	0.43	(0.30–0.63)

[1] Data in **Table 9** were analyzed by a meta-analysis [54–58] in a random effects model by the DerSimonian-Laird method [56, 59]. The meta-analysis was performed using the EZR [60] graphical user interface for R software (The R Foundation for Statistic Computing, version 2.14.0).

[2] Risk ratio (RR) was defined as: RR = $(a/A)/(b/B)$, where a is the number of rotted fruits in the fungicide application, A is the total number of fruits in the fungicide application, b is the number of rotted fruits in the control, and B is the total number of fruits in the control. RR is the ratio of the probability of disease development in the exposed group to that of the unexpected group [61]. In evaluating the effect of a fungicide application, a low RR indicates high effectiveness. The variance of RR (V) and the 95% confidence interval (95% CI) were defined as follows: $V = (1/a-1/A + 1/b-1/B)$, 95% CI = RR × exp (±1.96 × $V^{1/2}$). A prevention effect is recognized if the 95% CI of the combined risk ratio is <1.0.

Table 10. The efficacy of benzimidazoles, iminoctadine triacetate, and the tank-mix of two materials for the control of green mold in very early maturing satsuma mandarin orange.[1]

treatment. Only exception is Ogi-1 (2005), where efficacies of all treatments, including the combined spray, were low. Given the lack of efficacy of benomyl treatment, further examination of the BRS population may need for this location.

7.3. Tank-mix combination improves resistance to precipitation and aftereffects

If the growing area is large (i.e., several hectares or more), application of preharvest fungicide for postharvest rot control can be difficult. Maturity timing among outdoor Satsuma mandarin orange cultivars varies; thus, harvest can start from mid-September for very early maturing cultivars and continue to late November for the others. Therefore, a long residual effect is often a desired aspect of the preharvest fungicide. The combined spray of benomyl and iminoctadine triacetate applied 3 weeks prior to the harvest showed a sufficient level of green mold rot prevention (**Figure 7**). Also, it appeared that the effect of the combined treatment seemed not to be affected by rain. A sufficient level of efficacy was achieved even with the case where cumulative precipitation after spraying was high, e.g., about 150 mm (**Figure 8**). Thus, this combined spray has desirable a long residual activity and rain fastness. Further studies are needed to examine the residual effect under artificial precipitation to examine rain fastness of the combined spray.

7.4. Increased cost of fungicides using the tank-mix combination

As of 2016 in Saga prefecture, the costs of 500 L of benomyl is 1225 yen, and that of iminoctadine triacetate is 1662 yen, thus, the combined application costs 2887 yen. The cost increases for the combined application; however, as noted previously, the presence of rotten fruits will result in a substantial negative impact on the responsible grower as well as the related production area. Therefore, the benefits of risk mitigation far exceed the relatively small increase in the cost of fungicides. Moreover, the synergistic effect reported in this study should support growers' decision to apply these two fungicides in combination, even with the increased cost.

Year	Study[1]	Actual RRR (%) in green mold compared with the control			Expected RRR (%)[2]	Actual RRR (%)/ expected RRR (%)[3]
		Benomyl 125 μg/mL	Iminoctadine triacetate 125 μg/mL	Iminoctadine triacetate 125 μg/mL+ benomyl 125 μg/mL		
1999	Ogi-1	1.8	1.8	85.7	3.6	23.8
1999	Ogi-2	67.9	0	94.6	67.9	1.4
1999	Ogi-3	87.5	0	94.6	87.5	1.1
2000	Tara	0	0	76.5	0	∞
2000	Taku	46.5	0	90.7	46.5	2.0
2003	Taku-1	37.5	58.8	91.3	74.3	1.2
2003	Taku-2	3.8	96.3	78.8	96.4	0.8
2003	Taku-3	91.3	87.5	78.0	98.1	0.8
2004	Tara-2	52.9	27.2	73.6	65.7	1.1
2004	Chinzei-1	0	0	0	0	Not estimable
2005	Ogi-1	18.5	44.4	48.1	54.7	0.9
2005	Ogi-2	28.6	42.9	71.4	59.2	1.2

[1] See **Table 9**. Original data in the table are from [1], adapted with a permission from the authors.

[2] Determined using the formula $E = X + [Y(100-X)]/100$, where X = the percent control in plots treated with benomyl and Y = the percent control in plots treated with iminoctadine triacetate.

[3] Actual relative risk reduction (%) of iminoctadine triacetate 125 μg/mL + benomyl 125 μg/mL related to the expected relative risk reduction (%).

Table 11. Comparison of the expected and actual relative risk reduction percentages among benomyl, iminoctadine tiacetate, and a tank-mix of two materials.

7.5. Tank-mix combination is effective against green mold in varieties other than Satsuma mandarin orange

Superior disease-control effects of combined agents on green mold have been confirmed not only in Satsuma mandarin orange but also in mid-to-late-maturing citruses, such as Shiranui ((*C. unshiu* × *C. cinensis*) × *C. reticulata*) cultivated both outdoors and indoors [63].

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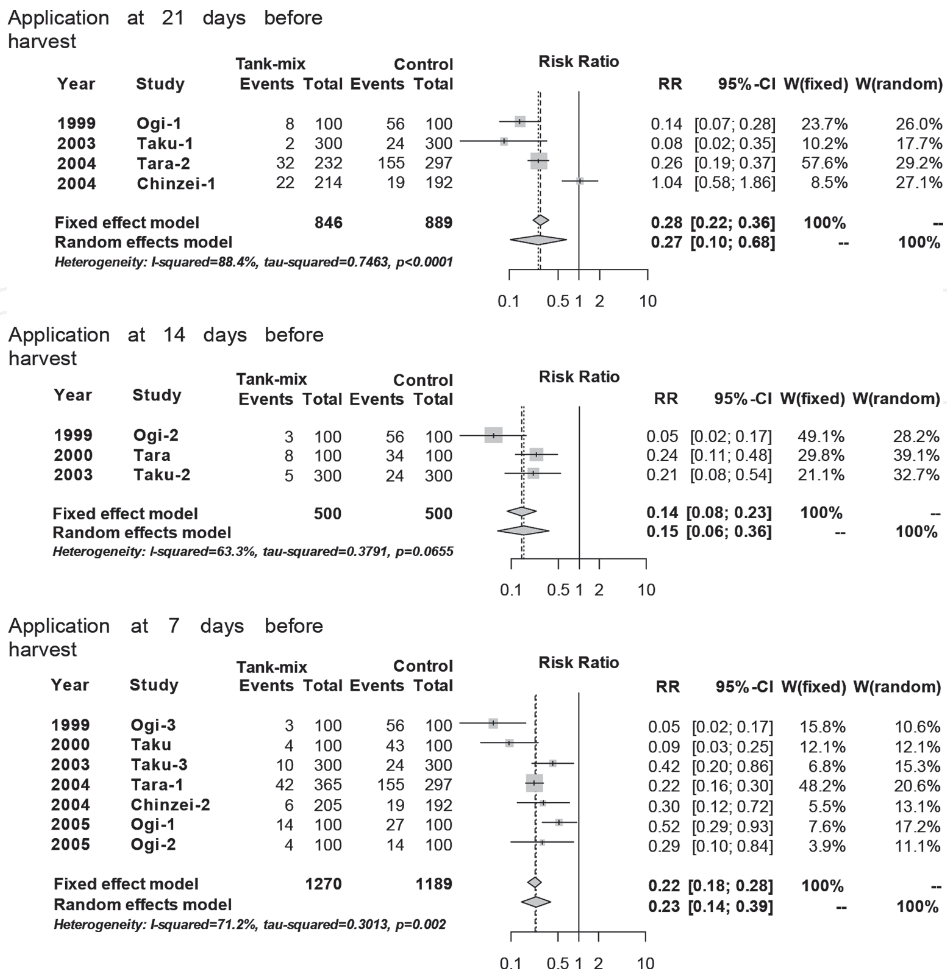

Figure 7. The forest plots (see **Figure 5**) represent the integrated meta-analysis of effect of the tank-mix application of benomyl and iminoctadine triacetate on green mold of very early ripening Satsuma mandarin orange different times. Data were analyzed by a meta-analysis in a random effects model using the DerSimonian-Laird method [56, 59]. The meta-analysis was performed using the EZR [60] graphical user interface for R software (The R Foundation for Statistic Computing, version 2.14.0).

7.6. The tank-mix combination is also effective against other postharvest diseases

Presently, in addition to green mold (*P. digitatum*), BRS of *Aspergillus* isolates are known, which is a serious disease-affecting greenhouse Satsuma mandarin oranges in warm temperatures. Against BRS of *Aspergillus* and anthracnose (*Colletotrichum* species), which results in rapidly increasing damage in September and October, an application of benzimidazoles is not sufficient. However, similar to observations with green mold, benzimidazoles combined with iminoctadine triacetate shown some favorable results [64–66].

7.7. Development and evaluation of a mixed iminoctadine triacetate/thiophanate-methyl agent

For some growers, properly mixing two fungicidal materials can be challenging; thus, the production of an easily administered premixed agent can be beneficial. In fact, the outcomes discussed in this chapter resulted in a development of a premixed product of iminoctadine triacetate and thiophanate-methyl (flowable), which was registered in December 2006.

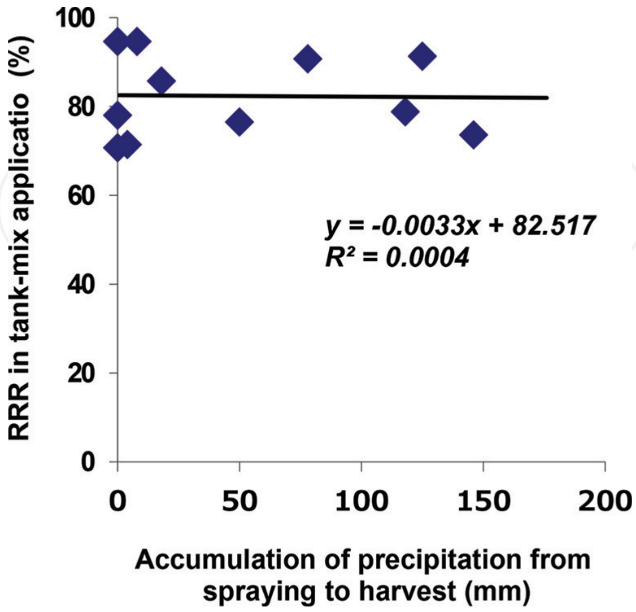

Figure 8. Influence of the tank-mix combination of benomyl 125 μg/mL and iminoctadine triacetate 125 μg/mL on the control of green mold in very early ripening Satsuma mandarin with respect to the accumulation of precipitation.

A pre-mixed product of 15.7% iminoctadine triacetate and 26.2% thiophanate-methyl is available in Japan. It was first registered in 2006 under the brand name Beftopsin flowable (Nippon Soda Co., Ltd.). Even in conditions of resistance, and as shown in the previous studies, synergistic effect is observed. Citrus diseases covered on the label are green mold, blue mold, stem-end rot, Alternaria rot, Aspergillus rot, sour rot, and anthracnose.

Limited studies have compared this pre-mixed product with the combined use of benzimidazoles and iminoctadine triacetate, but representative cases are described in **Table 12**. This mixed product had a favorable effect on green mold and as well as on anthracnose. Based on the RRR value comparison, its effectiveness was higher compared to that of the combined spray of iminoctadine triacetate and thiophanate-methyl. This may be attributed, at least in part, to the miniaturization of component particles by creating a flowable agent as well as the impact of an added auxiliary agent. Irrespective of the cause, the mixed product was a case of successful integration of two modes of action, which resulted in better efficacy than a combination of two solo materials and exhibiting an expanded range of target diseases.

Since this mixed product has better penetration and locally systemic effects, addition of acaricides, pesticides, and even fungicides other than benzimidazoles as a tank mix partner raised a concern on a coloring disorder in Satsuma mandarin orange fruit. However, none of mixtures did not result in symptoms of phytotoxicity (**Table 12**). Although the sample size is small, the combined use with other chemicals did not decrease the control effects (**Table 12**). In the future, we will continue to evaluate the effectiveness of this combined product on a number of crop varieties, both outdoors and indoors. Additional work is needed to determine the most effective use of this agent, such as determination of the optimum timing of spraying.

Mixed agent/+other compound	Active ingredient (%)	Rate applied	Investigated fruits	Rotted fruit		Total rotted fruit (%)	Discoloration on the fruit surface
				Green mold	Anthracnose		
Control			271	43	33	28.0	–
Tank-mix combination of iminoctadine triacetate and thiophanate-methyl			220	8	8	7.3	–
Mixed agent of iminoctadine triacetate and thiophanate-methyl (flowable)			113	1	0	0.9	–
+Milbemectin wettable powder (acaricide)	2	10 μg/mL	306	1	2	1.0	–
+Etoxazole flowable (acaricide)	10	50 μg/mL	232	1	2	1.3	–
+Spirodiclofen flowable (acaricide)	30	75 μg/mL	291	0	3	1.0	–
+Chlorfenapyr flowable (pesticide)	10	25 μg/mL	322	1	9	3.1	–
+Acetamiprid water soluble powder (pesticide)	20	100 μg/mL	301	3	4	2.3	–
+Acetamiprid liquid formulation (pesticide)	18	90 μg/mL	325	2	4	1.8	–
+Bifenthrin wettable powder (pesticide)	2	20 μg/mL	188	0	1	0.5	–
+Fosetyl wettable powder (fungicide)	80	2000 μg/mL	287	2	3	1.7	–
+Kresoxim-methyl wettable powder (fungicide)	50	10 μg/mL	234	0	4	1.7	–

[1] Chemicals were sprayed on October 6th, 2006, and fruits were harvested on October 16th. Harvested fruit were artificially damaged by rolling treatment at a slope of 5 m on concrete. Rotted fruit incidence was evaluated during storage for 30 days under the 98% relative humidity.

Table 12. The effect of the mixture of iminoctadine triacetate, thiophanate-methyl (flowable), and another material on postharvest diseases of very early maturing Satsuma mandarin orange.[1]

8. Conclusions

In this chapter, current understandings on increased cases of BRS of *P. digitatum*, the causal agent of green mold of citrus, and the synergestic efficacy of the tank-mix combination of two fungicides, benzimidazole and iminoctadine triacetate, against postharvest development of green mold based were summarized. Using the tank-mix combination of fungicides, two independent selection pressures by two fungicides are simultaneously applied to pathogens. To the best of our knowledge, iminoctadine triacetate–resistant *P. digitatum* [67] has not been found in Japan. However, it is necessary to carefully monitor how these selection pressures result in the changes of the sensitivity of pathogens to benzimidazoles and iminoctadine triacetate.

Because of superior effects on postharvest rot shown in various studies, the tank-mix of benzimidazole and iminoctadine triacetate is widely used in citrus-producing areas of Japan. Additionally, the usage of a newly developed iminoctadine triacetate + thiophanate-methyl wettable powder (flowable) has increased since its introduction. Although this chapter focused exclusively on the effectiveness of fungicides, the mechanism of observed synergistic effects is not clear and warrant further investigations. In addition, other factors that can influence the effectiveness of fungicide application, such as the spray volume and nozzle selection need to be evaluated. As shown in **Figure 2**, many factors are associated with postharvest diseases and the preharvest spray of a fungicide alone cannot control the onset of disease. Precise verification of causal factors for rot is necessary to develop comprehensive and appropriate countermeasures against these factors. The use of technology to maintain the freshness of harvested fruits is another future challenge [68].

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